IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Jean E.F. RIVIER et al.	
Serial No.: 10/763,935	
Filed: January 22, 2004	Group Art Unit: 1654
Title: CRFR1 SELECTIVE LIGANDS	Examiner: Thomas Sweeney Heard
Attorney Docket No.: 73933/5193	Confirmation No. 5057

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Dear Sir:

I, JEAN E.F. RIVIER, declare as follows:

1. I have a Ph.D. in Organic Chemistry from the University of Lausanne, Lausanne, Switzerland, and was employed in the Neuroendocrinology Laboratory at The Salk Institute for Biological Studies from about June, 1970 until the establishment of the Peptide Biology Laboratory at The Salk Institute, now re-named the Clayton Foundation Laboratories for Peptide Biology, where I am a professor and presently in charge of and responsible for all peptide syntheses. I have authored several hundred papers relating to synthetic peptides and peptide syntheses. I served as Chairman of the 11th American

Peptide Symposium in July 1989, and I was served as President of the American Peptide Society from June 1993 to June 1995.

2. During the course of my employment at The Salk Institute, I have directed and/or participated in syntheses of many thousands of biologically active peptides, including those set forth in detail in the above-identified U.S. patent application, of which I am the first-named inventor.

3. The 41-residue amino acid sequence of ovine CRF (oCRF) was first determined at the Salk Institute in 1981 in the Laboratories for Peptide Biology. Thereafter, the 41-residue amino acid sequence of rat CRF (rCRF) was determined in the same laboratories at the Salk Institute, which was subsequently ascertained to also be the 41-residue amino acid sequence of human CRF (hCRF). My laboratory has been synthesizing analogs of ovine and human CRF continuously since 1981 for over 25 years.

4. As a result of these many CRF analogs that have been synthesized and tested over this lengthy span of time, we have been able to form opinions that the three-dimensional structure of the CRF molecule is such that certain conservative substitutions at various particular locations in the 41 amino acid residue chain of the CRF molecule do not have a significant effect upon the binding of the ligand to the receptor.

5. Following the discovery of two different CRF receptors which participate in the G-protein-coupled response of cells, which receptors are now referred to as CRFR1 and CRFR2, our lab undertook a search for ligands that would bind selectively to one of these receptors with an affinity substantially greater than it would bind to the other. As a result of this research, the cyclic peptides disclosed in the above-identified application were discovered. Basically, it has been found that, if the 38-residue sequence extending from residues 4-41 of the CRF molecule includes certain critical substitutions, a ligand is created that exhibits binding to CRFR1 with an affinity substantially greater than it will bind to CRFR2, regardless of the presence of either the native residue or certain

conservative substitutions in a number of other locations throughout the amino acid sequence. For a CRF analog to exhibit this selective binding affinity, the following parameters have been found to be important:

(1) a covalent cyclizing bond exists between the side chains of the residues in positions 31 and 34 of the native sequence;

(2) the N-terminus is shortened by 3 residues from the native sequence and either tyrosine or an acyl group is added at the N-terminus;

(3) the C-terminus is amidated as in the native peptides; and

(4) a D-isomer which is either D-Phe, D-Leu or D-2-Nal is substituted at the 12 position of the native sequence.

6. I am informed that a claim substantially the same as that which follows was rejected as a dependent claim in the above-identified pending application on the basis of insufficient written description because of the number of different positions in the sequence that were open to substitution.

Claim 15

A 38-residue or 39-residue CRFR1 ligand cyclic peptide which binds to CRFR1 with an affinity substantially greater than it binds to CRFR2, which peptide has the following formula, or a nontoxic salt thereof:

(cyclo 31-34)Y_1-Pro-Pro-R_6-Ser-R_8-Asp-Leu-R_{11}-D-Phe-His-R_{14}-R_{15}-Arg-Glu-R_{18}-Leu-R_{20}-Nle-R_{22}-R_{23}-Ala-R_{25}-Gln-R_{27}-Ala-R_{29}-Gln-Glu-R_{32}-R_{33}-R_{34}-Arg-R_{36}-R_{37}-Nle-R_{39}-R_{40}-R_{41}-NH_2 wherein Y_1 is an acyl group having not more than 7 carbon atoms or is radioiodinated tyrosine; R_6 is Ile, Met or Nle; R_8 is Leu or Ile; R_{11} is Thr or Ser; R_{14} is CML or Leu; R_{15} is Leu or CML; R_{18} is Val, CML, Nle or Met; R_{20} is Glu or D-Glu; R_{22} is Ala or Thr; R_{23} is Arg or Lys; R_{25} is Asp or Glu; R_{27} is Leu or CML; R_{29} is Gln or Glu;

R_{32} is His, Aib, Ala, Gly, Leu, Gln or Glu; R_{33} is Aib or an L- or D-isomer of Ser, Asn, Leu, Ala, CML or Ile; R_{34} is Lys or Orn; R_{36} is Lys or Leu; R_{37} is CML or Leu; R_{39} is Glu or Asp; R_{40} is Ile, CML or Glu; and R_{41} is Ala, Aib or Ile; provided that D-β-(2-napthyl)alanine(D-2Nal) or D-Leu may be substituted for D-Phe.

7. The characteristic being claimed is that of the specificity of binding to CRFR1 as contrasted with binding to CRFR2. As pointed out in the specification these peptides will have a high binding affinity such that a ligand concentration of about 10 nanomolar or less will occupy, i.e. bind to, at least 50% of the receptor binding sites; this is expressed as a K_D of 10 nanomolar or less. At the same time, it was found that these ligands showing such a high affinity for CRFR1 would only weakly bind to CRFR2, thus exhibiting a substantially lesser affinity for CRFR2. While it is simply not reasonable to place absolute values on this biological effect, one of ordinary skill in this art would understand that the binding affinity of an order of magnitude less (i.e. 10 times less, or a K_D of at least about 10 times greater) would clearly constitute a substantially lesser affinity. For example, in Example 1, it was found that the cyclic analog showed high binding affinity of about 1.5 nanomolar for CRFR1, and only a weak binding affinity of about 224 nanomolar for CRFR2.

8. Our some 25 years of research in this area have convinced me that the particular conservative changes that are specified for certain residues in claim 15 would not alter the differential selective binding affinity that the ligand will show for CRFR1 as opposed to CRFR2. The specification is replete with a multitude of examples of CRF analogs meeting the criteria set forth in paragraph 5 above which exhibit high binding affinity for CRFR1 and a substantially lesser binding affinity for CRFR2 wherein there are variances in certain of the recited residues throughout the amino acid sequence. Specific reference is hereinafter made with regard to each of the substitutions enumerated in the above claim 15.

9. Over the past two decades, we have substituted the N-terminus of CRF analogs with a number of different acyl groups from formyl to benzoyl, or with radioiodinated tyrosine, and none of these substituents at the N-terminus has resulted in any significant change in the binding of the ligand to the CRF receptors.

10. With respect to position 6, the conservative substitution of isoleucine in hCRF by either methionine or norleucine has not been found to result in any significant change in the binding affinity of the ligand to the CRF receptors. Met is present at this position in other members of the CRF family.

11. With respect to the position 8 residue, leucine and isoleucine are found in hCRF and other members of the overall CRF family, and the inclusion of either one or the other has not been found to change the binding strength of the resultant ligand for the CRF receptor.

12. With respect to position 11, the conservative substitution of serine for the native residue threonine has been found to have no significant effect upon the binding of the ligand to CRF receptors.

13. As indicated above, it is felt important that the 12-position be occupied by a D-isomer; however, whether it is D-Phe or D-Leu (i.e. the D-isomers of the two residues found in the native peptides of this family) or D-2Nal, no significant change in the binding strength of the ligand to CRF receptors has been found to occur. With respect to positions 14 and 15, the presence of a C-alpha methyl group in the residue leucine (i.e. CML) was specifically shown in the peptides synthesized in Examples 5 and 5A to result in analogs having such selective binding affinity. With respect to position 18, the residues valine and threonine are found in the native members of the CRF family, and the conservative substitution of norleucine has long been often used in analogs we have synthesized with no significant effect on binding. Similarly, CML is also considered to be a conservative substitution for valine, and it was shown in Example 6B that such

substitution resulted in an analog exhibiting such differential selective binding of the ligand for CRFR1 compared to CRFR2.

14. Our laboratories have synthesized many CRF analogs where the D-isomer of glutamic acid was substituted in the 20 position for the naturally occurring L-isomer, and from our studies, I can say that this substitution does not cause any significant effect in the binding of a ligand to CRFR receptors.

15. The 21-position of human and ovine CRF is occupied my methionine, and one of the conservative substitutions of norleucine, leucine or CML has most often been made because they have not been found to exhibit any significant change in binding of the ligand to CRF receptors. Thus, in my opinion, a ligand that exhibits this differential binding affinity would do so regardless of which of these four residues was present at position 21.

16. Alanine and threonine in the 22-position, arginine and lysine in the 23-position, and aspartic acid and glutamic acid in the 25-position are the particular pairs of residues that are found in the native human and ovine CRF peptides. Throughout the past two decades, we have found that the presence of either one or the other of each of these pairs does not show any significant difference in the binding of the particular ligand to CRF receptors.

17. The conservative substitution of CML for leucine in the 27-position is not felt to have any effect on the differential selectivity, and such is shown in Example 5C. With respect to position 29, glutamic acid is a conservative substituent for glutamine, and one which is found in other members of the CRF family. The presence of either residue in the many analogs that we have made over the past two decades has not resulted in any significant change in the binding of the ligand to CRF receptors.

18. The tertiary structure of these ligands is significantly influenced by the covalent bond between the side chain of glutamic acid in the 31-position and the side

chain amino group of the residue (either lysine or ornithine) in the 34-position. As a result, it has been found that there is a great amount of latitude with respect to the character of the residues in positions 32 and 33 lying between those two residues where such side chain covalent linkage occurs. In this respect, the substitution of the residue His (found in native ovine and human CRF) by one of the other residues recited in claim 15 for position 32 (namely, Ala, Gly, Leu, Gln, or Glu), which residues are found in other members of the family in this position, has not be found to significantly effect binding to CRF receptors. The substitution of Aib (C-alpha methyl alanine) is likewise a conservative substitution which has occasionally been used and not found to change the differential binding affinity to CRF receptors.

19. Our studies with cyclic CRF analogs with a covalent bond between side chains in this region have shown that the presence of a D-isomer is not only tolerated but can be of some advantage in the 33-position. Accordingly, it is my opinion that, whether the 33-position is occupied by serine, asparagine, leucine, alanine or isoleucine (which residues appear in the various members of this overall CRF family), or the D-isomer thereof, does not affect the differential binding of these ligands. Similarly, the conservative substitutions of either C-alpha methyl leucine or C-alpha methyl alanine (see, for example, Example 4) does not affect the differential binding to the CRFR1 and CRFR2 of a ligand meeting this overall formula.

20. As previously indicated, it was found that the importance lay in the creation of a covalent bond between the side chains of the residues in positions 31 and 34, and the binding affinity of the resulting ligand is not affected by whether the side chain amino group is part of a lysine or an ornithine residue.

21. It was earlier found that the substitution of the residue lysine in position 36 by leucine, which appears in this position in another member of this overall CRF family, has no significant effect on the binding of the ligand to the CRF receptors. Likewise, the

conservative substitution of CML for leucine in the 37-position was not felt to have any effect on the differential selectivity, and such is shown in Example 5D.

22. With respect to position 39, Glu and Asp are the pair of residues found in ovine and human CRF at this position, and analogs which we have made for the last two decades or more have shown that the presence of one or the other at this position has no significant effect upon binding of the ligand to the CRF receptor. Similarly, with respect to the 40-position, the presence of either Ile (the residue in this position for ovine and human CRF) or Glu (which is present in numerous other members of the overall CRF family at this position) has not resulted in any significant difference in the binding of the ligand to CRF receptors.

23. Finally, with respect to position 41 at the C-terminus, the presence of either Ala or Ile, the two residues that appear at the C-terminus of ovine and human CRF has never resulted in compounds that showed any significant differences in binding to the CRF receptors. Likewise, the alternative, conservative substitution of C-alpha methyl alanine in various CRF analogs has not resulted in any significant change in the binding of the resultant ligand to CRF receptors, as compared to the same ligand having Ala at the C-terminus.

24. One understands that it is of course impractical to synthesize and test every one of the CRF ligands that would be covered by the multiple substitutions which appear in claim 15 above. However, we have repeatedly synthesized and tested CRF analogs over the past 25 years or more incorporating the individual named substituents and found that these individual substitutions do not significantly change binding to CRF receptors. More importantly, we have made a multitude of CRF analogs that have incorporated 2, 3, 4, 5 or more of these substitutions of the hCRF molecule in a single analog, and we did not find any significant difference in the binding of the analogs to CRF receptors whether the analog contained none, one or a multitude of these conservative

substitutions, compared to that exhibited by a similar analog having all the residues from human CRF in those same positions. Therefore, I am comfortable to say that there would be no cumulative effect resulting from the inclusion of multiple of these conservative substitutions enumerated in claim 15 within a single ligand that would significantly detract from the differential selective binding that a ligand meeting the 4 criteria set forth in paragraph 5 would exhibit to CRFR1, in contrast to CRFR2, regardless of the number of such conservative substitutions which were present.

25. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

/Jean E.F. Rivier/

Jean E.F. Rivier, Ph.D.

Dated: March 18, 2008